Reference: *Biol. Bull.*, **138**: 184–193. (April, 1970)

NUCLEAR-CYTOPLASMIC RELATIONS IN THE MITOSIS OF SEA URCHIN EGGS. II. THE DIVISION TIMES OF WHOLE EGGS AND HAPLOID AND DIPLOID HALF-EGGS [1]

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The relationship of the duration of the first mitotic cycle of marine eggs to the ploidy and the cytoplasmic volume can be studied by fertilizing nucleate and anucleate egg fragments. Such fragments can be prepared by shaking (Hertwig and Hertwig, 1887), drawing newly-fertilized eggs rapidly through a narrow orifice (C. B. Metz, personal communication), centrifugation (Lyon, 1907, *cf.* Harvey, 1956), and microsurgery (Delage, 1898). The first two mechanical methods produce egg fragments of various sizes and also produce varying amounts of cellular debris depending on the vigor of the treatment. Tennent (1912) stated that cytoplasmic fragments prepared by shaking and subsequently fertilized cleaved with the same "tempo" as the whole eggs. Although normal plutei can be raised from anucleate fragments prepared by the shaking method, the possibilities of cell damage and even of undetected nuclear fragments led Boveri (1918) to question the usefulness of this technique for embryological experiments. The first postfertilization division of enucleated half and quarter sea urchin eggs prepared by centrifugation is considerably retarded as compared to the whole egg or nucleate half; however, the nucleate quarter-egg is also greatly delayed in its division (*e.g.*, Harvey, 1932, 1956). Since the centrifugation method stratifies cytoplasmic organelles, the results obtained are to some extent a reflection of the cytoplasmic composition of the different types of fragments (*cf.* Moore, 1938; Kojima, 1959).

Variable results have been reported by different workers who prepared egg fragments by cutting with microneedles. In general, the conclusions have been based on very small numbers of eggs, and the possibility of surgical injury has not always been ruled out. In an earlier study (Rustad and Rustad, 1960) we found that amputating approximately half of the cytoplasm after fertilization had no obvious effect on the division time and that nucleate fragments as small as one-

[1] Supported by Contract W-31-109-ENG (Report Number COO-78-184) with the U. S. Atomic Energy Commission. The experiments with *Arbacia* were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts. The techniques employed in this study were developed with the support of the U. S. Atomic Energy Commission while the authors were affiliated with the Department of Biological Sciences and the Institute of Molecular Biophysics of Florida State University, Tallahassee, Florida.

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thirtieth of the whole cell volume could be fertilized and would divide at approximately the same time as the whole eggs.

With improved techniques it has been possible to prepare adequate numbers of nearly equal-sized nucleate and anucleate half-eggs for a careful comparison of the division times of whole and haploid and diploid half-eggs. Thus, it has been possible to determine whether or not either ploidy or cytoplasmic volume influence the duration of the mitotic cycle.

Materials and Methods

Gametes were obtained from the sea urchin *Arbacia punctulata* (collected at Woods Hole, Massachusetts) by stimulating the animals with a 12 volt alternating current (Iwata, 1950; Harvey, 1953). *Lytechinus pictus* (shipped by air to Cleveland, Ohio, from the Pacific Biomarine Supply Co., P.O. Box 285, Venice, California 90293) were induced to shed gametes by injecting them with 0.55 *M* KCl (Tyler, 1949). The eggs from *Arbacia* were washed three times in filtered sea water; the eggs from *Lytechinus* were washed three times in "Instant Ocean" (Aquarium Systems, Inc., 1450 E. 289 Street, Wickliffe, Ohio). Agar (0.2 to 0.3% in 80% sea water or in 80% "Instant Ocean") was heated and poured into Syracuse watch glasses. After the agar gelled the dishes were filled with sea water or "Instant Ocean" and washed in it several times while being stored for at least 24 hours in a refrigerator. This extensive washing procedure appeared to be necessary in order to prevent fragility, crenation, and even partial activation of the eggs by soluble materials in the agar gel. If the agar was too soft, the eggs would sink into it during cutting, and if it was too hard, the eggs tended to roll out from under the microneedle. The microneedles were prepared freehand over a microburner from thin-walled 2-mm glass capillary tubing. A concentrated suspension of eggs was placed in the center of an agar-coated Syracuse dish and the eggs were cut freehand into "halves" which appeared equal in size when examined with a Zeiss or Wild dissecting microscope. The cutting procedure consisted of resting the tip of the microneedle on the agar at some distance from the egg and then pressing the needle slowly down (often with a slight sawing motion) until the cell membrane on each side of the egg fused with itself and the two halves separated. Eggs from some animals flattened considerably under the pressure of the microneedle, so it was possible to make the cut through the widest part of the cell and obtain approximately equal-sized "halves." Other eggs tended to flow upward beside the needle so that a continuous adjustment of needle position was needed in order to produce two spheres of apparently equal diameter. Eggs which had not lost most of their jelly coat during the washing procedures were usually difficult to cut. If the eggs from a particular animal were fragile or difficult to cut, eggs from another animal were used. Since the nuclei of unflattened eggs of *Arbacia* are sometimes difficult to see under a dissecting microscope, the nucleate and anucleate halves were usually separated immediately after cutting. Nuclei in eggs of *Lytechinus* were always easy to see, so the half-eggs could be separated later. The half-eggs were often more difficult to fertilize yet more susceptible to polyspermy than whole ones. Therefore, considerable caution had to be exercised in fertilizing. The number of eggs of each type with division furrows were counted during the division period.

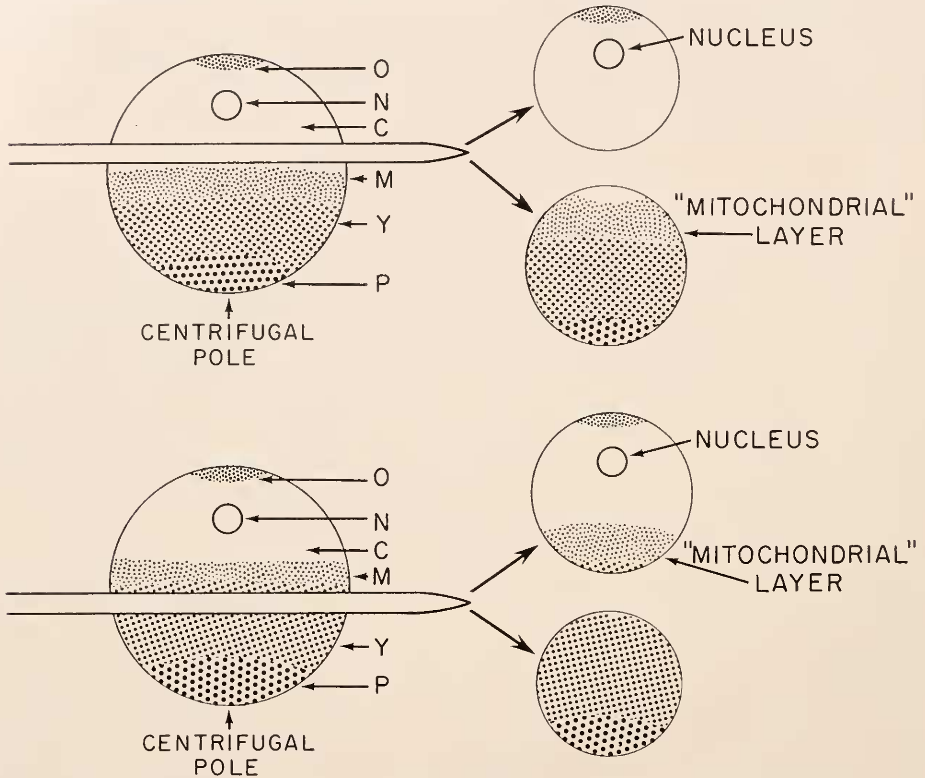

FIGURE 1. Microsurgery of eggs which have been stratified by centrifugation. Nucleate fragments lacking or containing the "mitochondrial layer" are formed when the microneedle passes on the centripetal or centrifugal side of the layer, respectively; Oil cap (O); Nucleus (N); Clear cytoplasm (C); "Mitochrondrial layer" (M); Yolk Granules (Y); Pigment granules (P).

In some experiments quarter-eggs were prepared by cutting nucleate or anucleate half-eggs into halves. In a limited number of experiments with *Arbacia* the cytoplasmic organelles of the eggs were stratified by centrifuging the cells at the interface between sea water and 1.1 *M* sucrose for 10 minutes at 10,000 times gravity (see Harvey, 1956). The eggs were quickly removed from the centrifuge tubes and mixed with sea water in the cutting dishes. These eggs were cut either on the centripetal or centrifugal side of the visible "mitochrondrial layer" before any redistribution of the stratified organelles could be detected (Fig. 1). Thus, both nucleate and anucleate cells either containing or lacking the "mitochondrial layer" were produced.

RESULTS

Whole eggs and diploid half-sized eggs divided at the same time while haploid half-sized eggs divided later. The distributions of division times of individual

eggs in a typical experiment are shown in Figure 2. In some experiments the number of late-dividing cells was somewhat larger in samples of diploid halves than of wholes; however, the times when 50% of the cells had cleaved did not appear to differ significantly.

The differences in times of 50% division of individual samples of more than 30 eggs or half-eggs (obtained from curves such as figure 2) have been analyzed. The data were derived from 796 diploid half-eggs in 25 separate experiments. The calculated average division time of the diploid half-egg is 1.3 ± 1.5 minutes earlier than the whole cell in the case of *Arbacia* (16 experiments with a total of 646 whole and 544 half-eggs) and 1.2 ± 0.8 minutes later than the whole cell

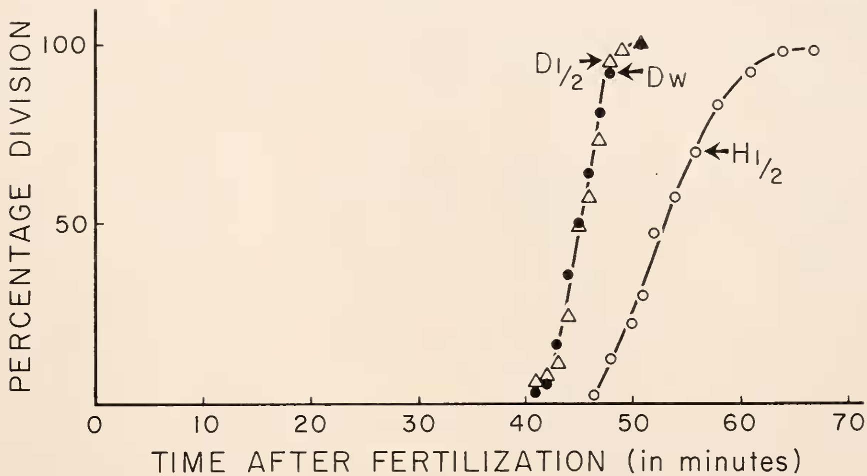

FIGURE 2. First division times of diploid whole eggs and of diploid and haploid half-eggs; Diploid whole eggs (D_W, solid circles); Diploid half-eggs ($D_{\frac{1}{2}}$, open triangles); Haploid half-eggs ($H_{\frac{1}{2}}$, open circles).

in the case of *Lytechinus* (9 experiments with a total of 330 whole and 252 half-eggs). Since the usual error of estimating the time of 50% cleavage of very large populations from single animals is approximately ± 1 minute, (*cf.* Harvey, 1956) these average differences cannot be considered significant. If the data from all of the experiments are averaged without regard to the genus of eggs, the calculated modification of division caused by removing half of the cytoplasm would not exceed one half minute (approximately 1% of the total duration of the mitotic cycle). Thus, amputation of half of the cytoplasm does not cause a significant mitotic delay.

The haploids divided later than either the whole cells or the diploid halves in every experiment (*e.g.*, Fig. 2). The delay of the time when 50% of the haploids cleaved was variable, ranging from 5 to 15 minutes. There did not appear to be any systematic correlation between the amount of delay and the genus of egg or period of the breeding season.

Only a limited number of fragments could be prepared from centrifuged eggs in any experiment, because it is difficult to control the plane of cutting and the

stratified organelles became redistributed rapidly by Brownian motion. When the cut was made on the centripetal side of the "mitochondrial layer" (Fig. 1), the nucleate fragments could be fertilized (elevating fertilization membranes) but did not divide within 24 hours. However, when the "mitochondrial layer" was included in the nucleate fragments (Fig. 1) they divided at approximately the same time as whole eggs. When anucleate eggs which lacked the "mitochondrial layer" were fertilized they divided some 40 to 70 minutes later than whole eggs. In contrast, haploid fragments which contained the "mitochondrial layer" were only delayed from 5 to 10 minutes compared to whole cells and were not detectably delayed compared to uncentrifuged haploids from the same animal. Thus, some factor found in the "mitochondrial layer" appears to be essential for a normal rate of division.

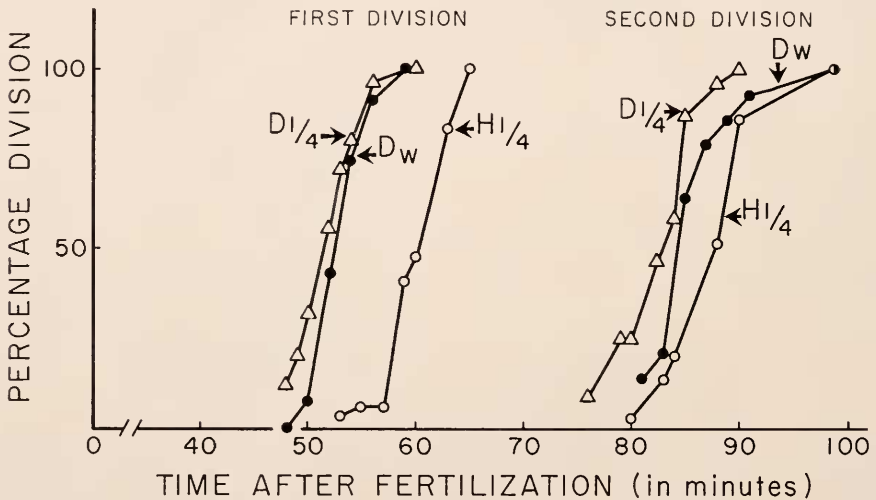

FIGURE 3. First and second division times of diploid whole eggs and of diploid and haploid quarter-eggs; Diploid whole eggs (D_W, closed circles); Diploid quarter-eggs ($D_{1/4}$, open triangles); Haploid quarter eggs ($H_{1/4}$, open circles).

Neither ploidy nor cell volume appeared to influence the duration of the second mitotic cycle. An extreme test involved comparing diploid whole cells with haploid and diploid quarter-sized eggs. The times of 50% first divisions of the whole eggs and the diploid quarter-sized cells did not differ by more than one minute, while the haploid quarter was delayed 7 minutes (Fig. 3, Table I). The durations of the second mitotic cycles of the whole embryos and the diploid quarter-sized ones were identical. The three-minute difference in the duration of the second cycle measured for the haploid quarter-sized embryos is not significant (Fig. 3, Table I). Hence, the duration of the second mitotic cycle is not influenced by a twofold variation in the ploidy or by a fourfold variation in the cytoplasmic volume.

Additional observations on the time of the fourth mitosis of whole, and both haploid and diploid quarter-eggs indicated that size had no obvious cumulative effect on the time of the fourth mitosis. Similarly, in haploid half-sized embryos the

total increase in the interval between fertilization and the fourth mitosis was equivalent to the delay of the first division rather than to an accumulation of four successive delays of similar magnitude.

The data indicate that cell volume does not influence the duration of the early mitotic cycles of the sea urchin egg and that even though haploid cells are delayed in their first mitosis, the subsequent cell cycles are not significantly retarded.

Discussion

Diploid whole and half-eggs

The present data on 796 half-eggs demonstrated that there is no significant difference between the times of division of 50% of a population of whole sea urchin eggs or 50% of a population of half-sized diploid eggs. Hence, the volume of cytoplasm does not affect the duration of the mitotic cycle of eggs of either *Arbacia punctulata* or *Lytechinus pictus.*

Table I

Division times of whole and quarter-eggs following fertilization

	Time of first division	Time of second division	Length of second division cycle
Diploid Whole	52 min	84 min	32 min
Diploid Quarter	51	83	32
Haploid Quarter	59	88	29

Previous workers using micromanipulators or cutting freehand were not able to obtain many half-eggs. For instance, Whitaker's (1929) conclusion that the diploid half-egg divided slightly sooner than whole cells depends on a total of 43 uncentrifuged diploid half-eggs cut during the course of 8 separate experiments, even though cells cut after centrifugation yielded similar data. The conflicting conclusions from other early investigations employing microsurgery were based on even smaller numbers of cells in each experiment (see Table II). Therefore, it seems that the conclusions of all previous workers were drawn from inadequate data.

Haploid versus *diploid eggs*

In each of 25 experiments in the present study involving more than 782 of each type of half-egg, the haploid half-eggs divided later than the wholes and diploid halves. This result was also obtained by some of the earlier workers who cut sea urchin eggs (see Table II) and may have been valid even when their measurements of smaller apparent differences between whole cells and diploid halves could be ascribed to sampling errors.

Although no data were presented, Tennent (1912) stated that haploid fragments obtained by shaking eggs of *Toxopneustes* (*Lytechinus*) had the same "tempo" of cleavage as diploid eggs. The classical crossfertilization experiments of Moore (1933) suggest in a very general way that the *type* of cytoplasm can limit the rate of cleavage. Eggs from *Strongylocentrotus* fertilized with sperm from the sand

TABLE II

A comparison of some previous studies concerning the relationship of ploidy and cytoplasmic volume to the cleavage time of echinoderm eggs

Method	Worker	Conclusion	Genus	Remarks
		Regarding duration of division cycle*		
Cut	Delage (1898)	$D_W < D_{\frac{1}{2}} < H_{\frac{1}{2}}$	*Strongylocentrotus*	12 haploid eggs and no quantitative data presented.
Shake	Tennent (1912)	$D_W = D_{\frac{1}{2}} = H_{\frac{1}{2}}$	"*Toxopneustus*" (*Lytechinus*)	No quantitative data presented.
Cut	Tennent, Taylor and Whitaker (1929)	$D_W < D_{\frac{1}{2}} < H_{\frac{1}{2}}$	*Lytechinus*	Many eggs cut, but only about 45% cleaved. Daily data on cleavage time of whole eggs were not reported.
Cut	Whitaker (1929)	$D_{\frac{1}{2}} < D_W < H_{\frac{1}{2}}$	*Arbacia*	43 haploid eggs. (Same conclusion with cut-centrifuged eggs):
Centrifuged	Harvey (1932)	$D_W \cong D_{\frac{1}{2}} \ll H_{\frac{1}{2}}$	*Arbacia*	Centripetal diploid quarters even slower. Furrows do not form on haploid half until many nuclei are present.
Cut	Moore (1933)	$D_W \cong D_{\frac{1}{2}} \cong H_{\frac{1}{2}}$	a) *Dendraster* ♂ x ♀ b) *Strongylocentrotus* ♂ x *Dendraster* ♀	One haploid sand dollar egg- 3 haploid eggs. Sea urchin-sand dollar "hybrids."

* D_W = Diploid whole egg; $D_{\frac{1}{2}}$ = Diploid half-egg; $H_{\frac{1}{2}}$ = Haploid half-egg.

dollar *Dendraster* divided at about the same time as normal eggs from *Strongylocentrotus* (*ca.* 95 min), and similarly, eggs from *Dendraster* fertilized with sperm from *Strongylocentrotus* cleaved at approximately the same time as the normal sand dollar eggs (*ca.* 55 min). These experiments with whole eggs suggest that the control of division time is either by the female pronucleus or by the cytoplasm, but not by the male pronucleus from the other order of echinoderm. Moore (1933) also fertilized four enucleated eggs fragments but the data seem insufficient to draw any conclusions concerning the normal nuclear-cytoplasmic relations in the timing of mitosis (see Table II). Thus, there appear to be no adequate data on any genus to contradict our general conclusion that first division of haploid half-eggs occurs later than that of either whole cells or diploid half-cells.

Centrifuged eggs

Nucleate fragments were prepared by cutting on the centripetal and centrifugal sides of the visible "mitochondrial layer" of the stratified eggs. The nucleate cells without the "mitochondrial layer" elevated fertilization membranes but did not divide, while those containing it divided at approximately the same time as whole eggs. This result agrees with results of studies in which half- and quarter-eggs were prepared by centrifuging until half- and/or quarter-eggs were pinched off as a result of density differences within the stratified eggs (reviewed by Harvey, 1956; see also: Moore, 1938; Kojima, 1959). Diploid half-eggs (from the centripetal half) divide relatively normally but smaller fragments which do not contain enough of some components found in or near the "mitochondrial layer" are either delayed many hours in their division or are unable to divide.

The division of haploid cells lacking the "mitochondrial layer" was found to be considerably retarded, as is also known to be true of such halves prepared directly by centrifugation (*e.g.*, Harvey, 1956). However, when anucleate fragments containing the "mitochondrial layer" were fertilized the division was only slightly retarded compared to whole eggs and no distinction could be made between the division times of these haploid cells and ones which had been prepared from uncentrifuged cells. Since removing the "mitochondrial layer" merely delayed the division of haploid centrifugal cells (rather than blocking it as in the case of the diploid centripetal cells) it would appear that some substance which is essential for division is found in the "mitochondrial layer" and is also present in a limited quantity in the more centrifugal parts of the cell. Moore (1938) studied the removal of a "cleavage substance" from the centripetal half of sand dollar eggs by centrifugation. Kojima (1959) suggested a correlation between the ability or inability of diploid fragments to divide, and the presence or absence of granules, which could be vitally stained with neutral red (as lysosomes are in some cells). Mitochondria are found throughout centrifuged eggs; however, there is a preferential accumulation of mitochondria with a clear matrix in the centrifugal half and those with a dense matrix in the centripetal half (Geuskens, 1965).

The postfertilization respiration rate of the haploid centrifugal fragment is only about half as great as that of the diploid centripetal fragment containing the "mitochondrial layer" (Shapiro, 1935). The present experiments clearly demonstrate that the "mitochondrial layer" contains some substance required for division which is not present in the more centrifugal parts of the cell in adequate abundance to permit a normal division rate. However, we do not know whether this "cleavage substance" is found in lysosomes, mitochondria or some unidentified structure.

Later divisions

The fact that the normal durations of the second, third, and fourth mitotic cycles are quite similar (*e.g.*, Harvey, 1956) indicates that the division rate is not strongly dependent upon cytoplasmic volume. Although the first mitosis occurred later in haploid than in diploid half-eggs in the present experiments, there was no comparable relay in the next three mitoses. The duration of the second mitotic cycle was not significantly influenced by either ploidy or by a fourfold reduction

of cytoplasmic volume. It was not possible to measure successively each of the later division times with the same precision as the first and second. However, if the second, third, and fourth mitotic cycles of the haploid cells had been delayed as much as the first, the cumulative delay by the time of the fourth division would have been 20 to 60 minutes. Delays of such magnitude were never encountered. Therefore, notwithstanding a delay of the first division of haploid cells, the amount of nuclear genetic material *per se* does not seem to limit the rate of mitosis. The delay of the first mitosis of a half-egg containing only a male pronucleus appears to be associated with the initiation of some activity associated with the first nuclear division cycle. Since such activities as protein synthesis can be activated artificially in anucleate eggs (reviewed by Tyler, 1966), it seems unlikely that the female pronucleus *per se* controls the initiation of cytoplasmic activities. It seems more probable that the division delay occurs in the initiation of, or the early events of the nuclear replication cycle. The DNA of the male pronucleus may remain compactly oriented and unable to replicate for a longer time when fusion with the larger more diffuse female pronucleus does not occur. Alternatively, the female pronucleus may contain enzymes and/or precursors for chromosomal duplication. Whatever factors limit the rate of the first but not later mitoses of the haploid egg, neither cytoplasmic volume nor ploidy appears to have any significant role in regulating the rate of cell division of sea urchin eggs.

Summary

Sea urchin eggs were cut into nucleate and anucleate halves. Following fertilization, the diploid half- and quarter-cells divided at the same time as whole cells, and the haploid half- and quarter-cells divided significantly later. However, there was no comparable increase in the duration of the second, third and fourth mitotic cycles of the haploid cells. Hence, the delay of the first mitosis of the haploid half-cells was attributed to the special consequences of the male pronucleus not fusing with a female pronucleus, and it was concluded that neither the cytoplasmic volume nor the ploidy of sea urchin eggs have significant effects on the timing of the mitotic cycle. Cutting experiments on eggs whose cytoplasmic organelles had been stratified by centrifugation suggested that some factor concentrated in the "mitochondrial layer" is essential for the maintenance of normal mitotic rates.

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